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P01/7700 0.00-0325968.6

Request for grant of a patent

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- 6 NOV 2003

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1. Your reference — N.87980 GCW/AB

2. Patent application number *(The Patent Office will fill this part in)* — 0325968.6

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

UCL BIOMEDICA PLC
c/o Finance Division
University College London
Gower Street, London, WC1E 6BT

Patents ADP number *(if you know it)* — 08748204001

If the applicant is a corporate body, give the country/state of its incorporation — UNITED KINGDOM

4. Title of the invention — IMPROVEMENTS RELATING TO SEMEN PRESERVATION

5. Name of your agent *(if you have one)* — J. A. KEMP & CO.

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

14 South Square
Gray's Inn
London
WC1R 5JJ

Patents ADP number *(if you know it)* — 00260001

6. Priority: Complete this section if you are declaring priority from one or more earlier patent applications, filed in the last 12 months.

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. Divisionals, etc: Complete this section only if this application is a divisional application or resulted from an entitlement dispute (see note f)

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8. Is a Patents Form 7/77 (Statement of inventorship and of right to grant of a patent) required in support of this request? — YES

Answer YES if:

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b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.

Otherwise answer NO (See note d)

Patents Form 1/77

9. Accompanying documents: A patent application must include a description of the invention. Not counting duplicates, please enter the number of pages of each item accompanying this form:

Continuation sheets of this form	-
Description	28
Claim(s)	5
Abstract	1
Drawing(s)	3 ONLY

10. If you are also filing any of the following, state how many against each item.

Priority documents	-
Translations of priority documents	-
Statement of inventorship and right to grant of a patent (Patents Form 7/77)	-
Request for a preliminary examination and search (Patents Form 9/77)	1
Request for a substantive examination (Patents Form 10/77)	-
Any other documents (please specify)	-

11. I/We request the grant of a patent on the basis of this application.

Signature(s) A. BENTHAM J.A. KEMP & CO. Date 6 November 2003

12. Name, daytime telephone number and e-mail address, if any, of person to contact in the United Kingdom

BENTHAM, Andrew
020 7405 3292

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IMPROVEMENTS RELATING TO SEMEN PRESERVATION

Field of the Invention

This invention relates to modulators of the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) present in mammalian semen. More specifically, the invention relates to the use of such modulators in improving the survival of cooled and/or cryo-preserved sperm and to predicting the suitability of a semen sample for cooling and/or cryopreservation, with a view to later use in assisted conception/reproductive techniques, in particular *in vitro* fertilisation (IVF), artificial insemination (AI) and/or intra-uterine insemination (IUI), in humans or livestock mammals. It also relates to means for improving the suitability of semen samples for use in such techniques.

Background of the Invention

The techniques of cooling and cryopreservation of semen are used to store semen at temperatures used for assisted conception/reproductive techniques, in particular *in vitro* fertilisation (IVF), artificial insemination (AI) and/or intra-uterine insemination (IUI). There are, however, difficulties associated with such techniques. In particular, when cooled or frozen semen samples are raised back to temperatures used in assisted conception/reproductive techniques, in particular IVF, AI and/or IUI, the sperm that they contain may become non-viable. The enzyme 11β-HSD converts cortisol to its inactive form, cortisone. Nacharaju *et al* (1997) have noted the presence of 11β-HSD in human semen and suggest a role for 11β-HSD in maintaining sperm development and function.

Previous work has shown that 11β-HSD activity in female follicular fluid is involved in determining the success of the outcome of assisted conception/ reproductive techniques. Generally, lower levels of 11β-HSD in follicular fluid are desirable to achieve successful assisted conception/reproductive treatment (WO 94/21815). It has also been shown that there are modulators of 11β-HSD activity in

female follicular fluid which influence the activity and hence effects of 11β-HSD (WO 97/30175 and Thurston *et al* (2002a)). These modulators have been characterised to a certain extent but not identified in chemical terms. The modulators were found to elute into two fractions on a C18 column (Thurston *et al* (2002a)). The first modulator fraction eluted at from 0 to 20% methanol. It is hydrophilic and stimulates 11β-HSD activity. The presence of the stimulatory modulator(s) in female follicular fluid was found to decrease the chances of successful assisted conception/reproductive techniques (Thurston *et al* (2003a)). The second modulator fraction elutes at from 60 to 100% methanol. It is hydrophobic and inhibits 11β-HSD activity. The presence of the inhibitory modulator(s) in female follicular fluid was found to increase the chances of successful assisted conception/reproductive techniques.

Summary of the Invention

We have found two new groups of modulators of 11β-HSD activity in semen. These protect sperm from death induced by cooling and cryopreservation. The two modulator fractions elute in two different fractions on a C18 column. The first is from 50 to 75% methanol. The second is from 95 to 100% methanol. Both groups of modulators are therefore hydrophobic in nature. Both stimulate 11β-HSD activity in rat kidney homogenates.

More specifically, we found that modulators of 11β-HSD activity in semen which elute from a C18 column at 65% and 100% (v/v) methanol protect sperm from death after full cryopreservation and thawing.

We found that, when semen samples are cooled and rewarmed and/or cryopreserved and thawed, those with modulator levels exhibited higher levels

that high levels of 11β-HSD stimulator (as isolated from seminal plasma) in semen are preferred in the context of preserving semen for assisted conception/reproductive techniques, in particular IVF, AI and/or IUI. Secondly, the modulators identified in female follicular fluid elute into two bands: hydrophobic 11β-HSD inhibitors and hydrophilic 11β-HSD stimulators. In contrast, the current invention has shown the presence of two hydrophobic modulators in semen both of which stimulate 11β-HSD activity. We did not find semen containing inhibitors, whether hydrophobic or hydrophilic.

A disadvantage of assisted conception/reproductive techniques which utilize cooled and/or cryopreserved semen is that the sperm often tend to lose their viability and/or longevity when the semen sample is brought back up to a temperature suitable for assisted conception/reproductive techniques.

The present invention thus relates to methods of determining the presence of the new modulators of 11β-HSD activity in semen and thus the suitability of such semen for cooling and/or cryopreservation.

The invention also relates to a method of improving the survival of cooled or cryopreserved sperm. By providing a more optimal concentration of the new 11β-HSD modulators it will be possible to recover a greater proportion of viable sperm after cooling and/or cryopreservation. Due to the presence of the modulators of the present invention, the resuspended semen sample is more likely to be viable for use in assisted conception/reproductive techniques, in particular IVF, AI and/or IUI.

In humans, assisted conception/reproductive techniques are expensive procedures and can be psychologically traumatic for patients. Therefore, until success rates are improved it would be desirable to be able to identify those men who produce semen likely to remain viable after cooling and/or cryopreservation by virtue of the high concentration of the modulators of the present invention. This will enable couples in which the male partner has unsuitable semen to choose to avoid assisted conception/reproductive techniques, if they wish, and also to avoid the banking of

unsuitable semen for anonymous use. It will also make it possible to screen the seminal plasma of men recruited by assisted conception clinics for semen donation. Similarly, it will be possible to help individuals with unsuitable semen by providing more optimal concentrations of modulators.

An advantage of the current invention is therefore to facilitate a good recovery of viable sperm following cooling or cryopreservation, and so to increase the chances of successful assisted conception/reproductive techniques, in particular IVF, AI and/or IUI, with the warmed sperm.

Additionally, in the context of livestock, certain mammalian species' semen samples, for example pig and horse semen, are expensive, and some species' semen is more prone than that of others to losing viability when cooled/cryopreserved. The current invention is therefore economically advantageous in that it reduces costs by improving the chances of successful assisted conception/reproductive techniques, in livestock. It may also increase the number of offspring that can be obtained from particular high quality individuals that unfortunately happen to have sperm susceptible to a high rate of cooling-induced cell death.

The present invention will be applicable to any mammalian male and can be used not only in human assisted conception/reproductive programmes but also to increase the success of, for example, captive breeding programmes of endangered species or commercial breeding by assisted conception/reproductive techniques of livestock such as cattle, pigs and horses. It will also be applicable to rodent sperm, which will be of particular value in cooling/cryopreservation of the sperm of transgenic mice.

Accordingly, the invention provides a method of determining the suitability

activity in said sample; and

(b) assessing, from the level of 11β-HSD stimulator determined, the suitability of the semen sample for cooling and/or cryopreservation.

The invention also provides a method of improving the survival rate of sperm intended for cooling and/or cryopreservation, said method comprising:

(a) providing a sample of semen; and

(b) combining said sample of semen with an increased concentration of a hydrophobic stimulator of 11β-HSD activity.

The invention also provides a method of fertilizing an oocyte *in vitro* comprising contacting said oocyte with sperm obtained by a method of improving the survival rate of sperm intended for cooling and/or cryopreservation, said method comprising:

(a) providing a sample of semen; and

(b) combining said sample of semen with an increased concentration of a hydrophobic stimulator of 11β-HSD activity under conditions which allow fertilization of the oocyte.

The invention also provides a method of performing an assisted conception/reproductive procedure comprising contacting an oocyte with sperm obtained by a method of improving the survival rate of sperm intended for cooling and/or cryopreservation, said method comprising:

(a) providing a sample of semen; and

(b) combining said sample of semen with an increased concentration of a hydrophobic stimulator of 11β-HSD activity under conditions which allow fertilization of the oocyte.

The invention also provides a method of obtaining a hydrophobic product that improves the tolerance of mammalian semen to cooling and/or cryopreservation, comprising the steps of:

(a) providing a sample of semen;

(b) removing the seminal plasma from the sperm; and

(c) fractionating the seminal plasma of (b) to enrich for said product.

The invention also provides a product obtainable by fractionation of mammalian seminal plasma and having a stimulatory effect on 11β-HSD activity, which improves the tolerance of semen to cooling and/or cryopreservation.

The invention also provides the use of said product to improve the tolerance semen to cooling and/or cryoperservation.

The invention also provides the use of said product in the manufacture of a medicament for use in the treatment of inflammatory disease by increasing the survival of topically applied cortisol or cortisol already circulating within the bloodstream.

The invention also provides the use of said product in the manufacture of a medicament for use in the treatment of inflammatory disease by stimulating the production of cortisol from circulating cortisone by stimulation of 11β-HSD1.

Brief Description of the Drawings

Figure 1: Effects of fractions of seminal plasma eluted from a C18 column with different concentrations of methanol on 11β-HSD activity.

Figure 2: Variation in the effect of lipid extracted from seminal plasma on sperm viability following cooling to 5°C and rewarming to 39°C (mean +/- SEM; n=5; values with different superscripts differ P<0.05). SP1, SP2 and SP3 denote effects of seminal plasma samples taken from three male goats.

Detailed Description of the Invention

The present invention provides a method of determining the suitability of a sample of semen for cooling and/or cryopreservation. The invention also provides a method of improving the survival rate of sperm intended for cooling and/or cryopreservation. The invention further provides a method of obtaining a hydrophobic product that improves the tolerance of semen to cooling and/or cryopreservation.

Obtaining samples of semen

The first step in the methods of the current invention is obtaining a semen sample. Semen from any species can be selected and assayed for the presence of a hydrophobic stimulator of 11β-HSD activity. Semen samples are preferably collected by manual ejaculation methods, through use of an artificial vagina (AV), or electro-ejaculation. The method can be carried out with sperm from any mammal, notably human sperm and that of domesticated animals, especially livestock animals, as well as with sperm from wild animals (e.g. endangered species). Human sperm, bovine, equine, porcine and ovine sperm are more preferred.

Determining the level of hydrophobic stimulator of 11β-HSD activity

The second step in the method of determining the suitability of a semen sample for cryopreservation and/or cooling is to determine the level of hydrophobic stimulator of 11β-HSD activity in the sample of semen. By hydrophobic stimulator of 11β-HSD is meant a product that increases 11β-HSD activity. The product is hydrophobic in nature and preferably elutes from a C18 chromatography column at from 50 to 75% methanol or from 95 to 100% methanol. The hydrophobic stimulator may be an agonist or a cofactor of 11β-HSD activity.

The level of hydrophobic stimulator of 11β-HSD activity may be measured directly (e.g. by determining the amount or concentration of the hydrophobic

stimulator) or indirectly (by the level of 11β-HSD activity) as that will often be affected by the amount or concentration of hydrophobic stimulator present.

For example, the amount/concentration of hydrophobic stimulator may be measured through the conversion of a substrate of 11β-HSD (e.g. ^{3}H-cortisol or ^{3}H-corticosterone) to a product (e.g. ^{3}H-cortisone or ^{3}H-11-dehyrocorticosterone respectively) in the absence and presence of hydrophobic stimulator. This involves contacting the sample with the substrate, for example ^{3}H-cortisol, and measuring the conversion of the substrate to ^{3}H-cortisone by 11β-HSD. In such a case the higher the level of cortisone in relation to cortisol, the higher the activity of the enzyme and hence higher the level of hydrophobic stimulator present in the sample. The level of ^{3}H-cortisone and ^{3}H-cortisol can be measured by methods known *per se*. TLC/HPLC may be used to resolve ^{3}H-cortisol/^{3}H-cortisone followed by the quantification of ^{3}H-cortisol and ^{3}H-cortisone levels by liquid scintillation counting or preferably through use of a TLC radiochromatogramme scanner. This method will provide a direct measurement of enzyme activity, and for this reason is preferred. In a typical assay a concentration range of about 100nM of ^{3}H-cortisol maybe used, although a concentration ranging from 10nM to 1000nM or more can be used.

Alternatively, or in addition, a semen sample may be contacted with 11β-HSD present in another body fluid or cultured cells or another body derived substance for example homogenised animal organs (such as rat kidney). A suitable control assay may also need to be conducted to allow for 11β-HSD already and naturally present in the sample. The 11β-HSD used in the assay may be from an isolated or purified source or can be present in another human or animal body or body derived fluid for example kidney homogenates. The 11β-HSD used in the

Alternatively 11β-HSD activity can be measured by immunoassay or similar ligand binding techniques. This will provide an indication of the amount of the enzyme, which may be correlated to enzyme activity and from there to the amount/concentration of hydrophobic stimulator of 11β-HSD activity. For example a ligand (or antibody) capable of binding the enzyme could be used in immunoassay methods such as RIA or ELISA. In addition, the level of 11β-HSD enzyme or even its mRNA can be used as a measure of a modulator activity since some modulators may exert their effects at the level of mRNA transcription or translation.

The expression of the 11β-HSD enzyme can also be measured by immunochemistry using a monoclonal antibody. Such techniques will provide a measurement of the amount of 11β-HSD present, which can then be correlated to enzyme activity.

Although reference is made in the specification to determining levels of 11β-HSD (and its modulators) it will be understood from the foregoing that this also includes the indirect measurements mentioned above.

The presence of these modulators in semen increases the viability of sperm following cryopreservation and/or cooling, This is a surprising result due to the well known fragility of sperm following cryopreservation and/or cooling. Researches have shown the stresses associated with cryopreservation resulted in losses in fertility and/or viabilty. The present inventors have identified modulators of 11β-HSD function which when present in semen samples increases the viability of semen undergoing cyopreservation and/or cooling.

Assessing the suitability of the semen sample for cooling and/or cryopreservation

Once the level of hydrophobic stimulator of 11β-HSD activity has been measured, the result can be used to determine the suitability of the semen sample for cryopreservation and/or cooling. The level of 11β-HSD activity in the sample will

be directly affected by the hydrophobic stimulator. Therefore, 11β-HSD activity will be proportional to the level of hydrophobic stimulator and a measurement of the level of hydrophobic stimulator can thus be correlated back to the level of 11β-HSD activity.

It is desirable to add to sperm an amount or concentration of 11β-HSD stimulator derived from seminal plasma which, when assessed at a dilution of 10% by volume, can increase 11β-HSD activity by 100% or more relative to enzyme activity measured in the absence of the stimulator.

The current invention has found that high levels of 11β-HSD stimulator in semen are preferred for successful assisted conception/reproductive techniques, in particular IVF, AI and/or IUI. In particular, those subjects who have hydrophobic stimulator present in their semen at a predetermined threshold indicated above, or exceeding this threshold are preferred as suitable candidates for having their semen cooled and/or cryopreserved.

For example, pig serum generally cools/freezes relatively poorly compared, say, to human sperm where typical survival rates might be, for example 70-80%. In pig semen samples with low levels of hydrophobic stimulator, only around 20 to 60% of sperm typically survive cooling and/or cryopreservation and rewarming. We have found that figure is significantly increased when the semen either contains or is supplemented with high level of hydrophobic stimulator. Semen having the hydrophobic stimulator at or above the above mentioned threshold is more likely to undergo cooling and/or cryopreservation and still contain in excess of 70% viable sperm when the semen sample is brought back up to a temperature suitable for assisted conception/reproductive techniques.

modulator is only determined for one ejaculate decisions as to the suitability of that particular male may only be possible based on the results for that ejaculate, although determinations over several ejaculated samples may give a more general indication of the suitability of the semen to undergo cooling and/or cryopreservation.

By use of the present invention, it will be possible for clinics which perform assisted conception/reproductive techniques, in particular IVF, AI and/or IUI, to allocate resources more efficiently. Male subjects with low levels of a hydrophobic stimulator of 11β-HSD activity in their semen, and thus have semen unsuitable for cooling and/or cryopresevation, can be identified prior to freezing their semen, and the semen samples can be treated to improve its survival rate.

Improving the survival rate of sperm intended for cooling and/or cryopreservation

The current invention also provides methods of improving the survival rate of sperm intended for cooling and/or cryopreservation. In one aspect of the present invention, a semen sample intended for cooling and cropreservation is obtained by methods known in the art or detailed above. The semen sample is then combined with an increased concentration of a hydrophobic stimulator of 11β-HSD activity.

It would be desirable to add to sperm an amount or concentration of 11β-HSD stimulator (derived from seminal plasma) which ,when assessed at a dilution of 10% by volume, can increase 11β-HSD activity by 100% or more relative to enzyme activity measured in the absence of the stimulator.

This method may additionally comprise an initial step of removing the desirable sperm from its seminal plasma followed by combining the desirable sperm with an increased concentration of hydrophobic stimulator of 11β-HSD. Sperm can be isolated from semen samples using any sufficiently gentle isolation method that provides at least about 50% recovery of sperm, preferably 75% or more preferably 90% recovery of sperm.

In a preferred embodiment, sperm separated from its seminal plasma may be combined with seminal plasma from another individual known to have an increased amount of hydrophobic stimulator of 11β-HSD activity in his seminal plasma. Preferably this embodiment may apply to male animals other than humans.

The percentage sperm recovery using the centrifuge technique is approximately 90%. Percoll centifugation and Percoll 'swim up' may also be used to recover sperm cells. The percentage sperm recovered are approximately 75% and 50% for the Percoll centrifugation and Percoll 'swim-up' respectively.

The methods of the invention may be applied to any mammalian species. The semen may, for example, be of human origin, or of rodent, bovine, equine, porcine or ovine origin.

In the context of pig semen, the method of the invention preferably results in the survival of at least 40%, at least 50%, preferably at least 60% and more preferably at least 70% or more of the sperm following cooling and/or cryopreservation.

In the context of human semen, whose typical survival rate is higher (70-80%) in any case, the method of the invention preferably results in the survival of at least 85%, at least 90%, or at least 95% of the sperm following cooling and/or cryopreservation.

In the context of horse or sheep sperm, the method of the invention preferably results in the survival of at least 50%, at least 60% or at least 70% of the sperm following cooling and/or cryopreservation.

In the context of rodent (mouse or rat) sperm, the method of the invention preferably results in the survival of at least 60%, at least 70% or at least 80% of the sperm following cooling and/or cryopreservation.

In all species, the methods of the invention preferably result in sperm survival being increased significantly compared to a situation where the same individual's sperm is used, but without the benefit of the methods of the invention. The degree of improvement will vary from individual to individual within a species, and also from species to species. In species where sperm survival rates are already relatively high, e.g. in humans and cows the degree of improvement may be, say, at least 5%, at least 10%, at least 20%, at least 25% or at least 50%. However, in particular individuals with low sperm survival rates, the degree of improvement may be higher, e.g. 100% or more, or 200% or more. In species with relatively low sperm survival rates, for example pigs, sheep and horses, the degree of improvement may be, for example, at least 5%, at least 10%, at least 20%, at least 25%, at least 50%, at least 75% or at least 100% or more. In individuals with low sperm survival rates, the degree of improvement may be even higher, e.g. 200% or more.

Any of a variety of methods suitable for recovering cells from a suspension can be used to isolate the sperm, including for example filtration, sedimentation and centrifugation. In a preferred embodiment, the selected semen sample is aliquoted into 50ml tubes at volumes not exceeding about 27 ml, and preferably from between about 20 to 27ml. Centrifugation is carried out at ambient temperature at about 600 x g, for about 10 mins and then 3000 x g for 30 mins. Preferably the centrifugation step provides at least about 90% recovery of sperm from a semen sample.

For methods concerned with improving the survival rate of sperm intended for cooling and/or cryopreservation the pellet of sperm resulting from centrifugation is resuspended in the presence of an increased concentration of 11β-HSD stimulator. Typically, the concentration of 11β-HSD stimulator added to the pelleted sperm equates to an amount or concentration which, when assessed at a dilution of 10% by

volume, could increase 11β-HSD activity by 100% or more relative to enzyme activity measured in the absence of the stimulator.

The reconstituted sperm and hydrophobic stimulator of 11β-HSD activity may be cooled or frozen to 5°C and below. If the sperm is intended for use in assisted conception/reproductive techniques, in particular IVF, AI and/or IUI, the semen sample may be conveniently aliquoted into individual doses sufficient to achieve fertilization. The individual dose may vary from one species to the next and is either well-known or can be readily determined.

If being cryopreserved, prior to freezing, the sperm are generally allowed to equilibrate at about 5°C. Generally, the sperm are allowed to equilibrate for a period of from 1 to about 6 hours. The preferred time is species-dependent, 3 to 6 hours being typical for pig sperm.

Typically, semen samples are collected at body temperature e.g. 37°C for a human and around 37 °C for other mammals, and cooled to 5°C at 0.5°C/min. Samples are then equilibrated for between 0 to 6 hours to attain resistance to cold shock. This is dependent on species where human sperm need no equilibration and pig sperm require 3 to 6 hours. Sperm is then cooled from 5°C to -5°C at 6°C/min in the presence of cryoprotectants such as glycerol and egg yolk. Sperm are then cooled from -5°C to -80°C at 40°C/min and then plunged into liquid nitrogen to a final temperature of -196°C. The optimal freezing rates will vary between species.

Methods of obtaining a hydrophobic product of the invention

The present invention also encompasses hydrophobic compounds having a

separated into fractions which are enriched for the hydrophobic stimulator of 11β-HSD activity.

The Inventors have characterised fractions from boar semen which modulated the activity of 11β-HSD and which elute on a C18 column at either from 50 to 75% methanol or 95 to 100% methanol. These modulators are therefore hydrophobic and both stimulate 11β-HSD activity in semen. The C18 column which binds the modulators may be one which is available from Walters Chromatography (Hertford UK). The C18 column binds hydrophobic substances, and thus bind the modulators of the current invention since these are hydrophobic. The C18 column usually comprises silica with a poor volume of 0.5 to 1.5ml/g, such as 0.9 to 1.15ml/g. The column preferably has a poor diameter from 130 to 250A e.g. from 170 to 200A and/or a surface area of 150 to 250m^2/g e.g. from 190 to 220m^2/g. Generally the ligand bonded to such silica is octadecyldimethyl sibyl or the end capping groups are trimethyl sibyl groups.

A further embodiment of the current invention is the use of sperm, for which its survival rate following cooling and/or cryopreservation is improved by a method according to the current invention, in assisted conception/reproductive techniques, in particular IVF, AI and/or IUI.

Kits for use in detemining the suitability of a semen sample for cooling and/or cryopreservation

The present invention also provides kits for determining the suitability of a sample of semen for cooling and/or cryopreservation. Such kits include at least one reagent useful for the detection of a hydrophobic stimulator of 11β-HSD activity. Suitable reagents (for direct detection or determination of hydrophobic stimulator of 11β-HSD activity/concentration) include antibodies, or suitable ligand binding agents, against the modulator. Such antibodies and reagents may be linked to a label. Typical labels are those commonly used in immunoassay procedures, for example horseradish peroxidase (HRP). Alternatively, the kit may contain antibodies, or

other suitable ligand binding reagents against cortisol and/or cortisone. This may be suitable for indirect assays (measuring the level of 11β-HSD activity) such as determination of the cortisol or cortisone ratio or radiometric conversion of ^{3}H-cortisol to ^{3}H-cortisone. The kit may also contain standards, for example predetermined amounts of cortisone, cortisol and/or 11β-HSD, any or all of which may be labelled with a detectable label. The kit may also contain enzyme cofactors, for example, NAD^{+} or $NADP^{+}$.

The kit may comprise agents such as oxidised tetrazolium salts to serve as a colorimetric substrate for the reoxidation of the reduced NADPH. The change in optical density of the indicator shows that the appropriate wavelength may be directly proportional to the rate of reduction of the $NADP^{+}$ plus co-factor, which may in turn be directly proportional to 11β-HSD activity and hence proportional to the concentration of hydrophobic stimulator of 11β-HSD activity in the sample.

Therapeutics

Another aspect of the invention is a method of fertilization of oocytes *in vivo* using artificial insemination and/or intra-uterine insemination comprising contacting an oocyte with sperm obtained by a method of improving its survival rate of cooling and/or cryopreservation according to the invention under conditions which allow fertilization of the oocyte to form a zygote.

Thus, sperm obtained by a method of improving its survival rate of cooling and/or cryporeservation according to the invention may be used in the manufacture of a medicament for use in the fertilization of oocytes *in vivo* using artificial insemination and/or intra-uterine insemination.

NAD^+ dependent manner. 11β-HSD1 is also involved in this process but also catalyses the reverse process of the conversion of inactive cortisone to active cortisol.

It is known in the art that cortisol plays a role in the inflammatory response by directly acting to reduce inflammation. Thus, a hydrophobic stimulator of 11β-HSD activity identified by methods of the present invention may be used in the manufacture of a medicament for use in the treatment of inflammatory disease. The conditions of inflammation can therefore be improved by administration of such a hydrophobic stimulator of 11β-HSD activity. A therapeutically effective amount of a hydrophobic stimulator of 11β-HSD activity used in the treatment of inflammation may be given to a patient in need thereof.

In the invention, the hydrophobic stimulator of 11β-HSD activity may be used in the reduction of inflammation and may be administered in a variety of dosage forms. A preferred embodiment is to apply a formulation of the hydrophobic stimulator of 11β-HSD activity topically to a patient in need thereof in order to potentiate or lengthen the presence of naturally occurring cortisol at the site of inflammation.

The formulation of a hydrophobic stimulator of 11β-HSD activity used in the treatment of inflammation according to the invention will depend upon factors such as the nature of the exact stimulator of 11β-HSD activity.

A hydrophobic stimulator of 11β-HSD activity used in the treatment of inflammation according to the invention is typically formulated for administration with a pharmaceutically acceptable carrier or diluent. Preferably, the formulation will be a topical one. The pharmaceutical carrier or diluent may be, for example, an isotonic solution or together with the active substance, diluents, e.g. lactose, dextrose, saccharose, cellulose, corn starch or potato starch; lubricants, e.g. silica, talc, stearic acid, magnesium or calcium stearate, and/or polyethylene glycols; binding agents; e.g. starches, gum arabic, gelatin, methylcellulose, carboxymethylcellulose or polyvinyl pyrrolidone; disaggregating agents, e.g. starch,

alginic acid, alginates or sodium starch glycolate and, in general, non-toxic and pharmacologically inactive substances used in pharmaceutical formulations. Such pharmaceutical preparations may be manufactured in known manner, for example, by means of mixing, granulating and forming into a cream.

Suspensions and emulsions may contain as carrier, for example a natural gum, agar, sodium alginate, pectin, methylcellulose, carboxymethylcellulose, or polyvinyl alcohol. The suspensions or solutions for topical administration may contain, together with the active substance, a pharmaceutically acceptable carrier, e.g. sterile water, olive oil, ethyl oleate, glycols, e.g. propylene glycol, and if desired, a suitable amount of lidocaine hydrochloride.

A therapeutically effective amount of a substance used in the treatment of inflammation may be administered to a patient identified according to a method of the invention. The dose, for example of a hydrophobic stimulator of 11β-HSD activity, may be determined according to various parameters, especially according to the substance used; the age, weight and condition of the patient to be treated; and the required regimen. Again, a physician will be able to determine the required route of administration and dosage for any particular patient. A typical daily dose is from about 0.1 to 50 mg per kg of body weight, according to the activity of the hydrophobic stimulator of 11β-HSD activity, the age, weight and conditions of the subject to be treated and the frequency of administration. Preferably, daily dosage levels are from 5 mg to 2 g. That dose may be provided as a single dose or may be provided as multiple doses, for example taken at regular intervals, for example 2, 3 or 4 doses administered daily.

Materials and Methods

Seminal Plasma and Spermatozoa Samples

Ejaculates were collected from Large White stud boars. All boars were 1-1.5 years of age of proven fertility and undergoing regular semen collection for commercial artificial inseminations. All animals received the same diet.

An entire porcine ejaculate (sperm rich fraction and the gel fraction) was manually collected (King & MacPherson, 1973) from each of 3 individual boars and shipped to the laboratory at ambient temperature within 24 hours of collection. After centrifugation of the ejaculates for 10 minutes at 600 x g to sediment spermatozoa, the sperm pellet from each ejaculate was discarded. The remaining supernatant was then aspirated and centrifuged for 30 minutes at 3000 x g to pellet any remaining spermatozoa and debris. The supernatant (which was still opaque) was decanted and stored at -20°C until required for use.

The sperm rich fractions of a further 5 ejaculates, each from a different individual boar, were subsequently collected as a source of viable spermatozoa. Immediately following ejaculation, semen samples were diluted with washing buffer (447mOsm kg^{-1}; Cat: ZS993, IMV, France) to a concentration of $100x10^6$ sperm/ml, allowed to cool from 39°C to room temperature at approximately 0.2°C/min. The diluted semen was then shipped to the laboratory at ambient temperature within 24 hours.

Effects of Seminal plasma on Renal 11β-HSD Activities

The effects of the porcine seminal plasma from 3 independent boars on renal 11β-HSD activities were assessed using a modification of the radiometric conversion assay for glucocorticoid oxidation previously described in our laboratory (Michael *et al.*, 1993; Michael *et al.*, 1995). Male Sprague-Dawley rats (200-250g), housed in accordance with the UK Animals (Scientific Procedures) Act 1986, were allowed ad

libitum access to a standard rat chow diet and drinking water. Rats were sacrificed by cervical dislocation and kidney homogenates prepared using established methods (Sewell *et al.*, 1998; Thompson *et al.*, 2000). Renal homogenates were utilised as a source of $NADP^+$-dependent, 11β-HSD1 activity. For each assay, 1g of rat kidney (containing approximately equal volumes of cortex and medulla) was homogenised in 18ml hypotonic Tris-EDTA lysis buffer (Rusvai & Naray-Fejes-Toth, 1993; Sewell *et al.*, 1998; Thompson *et al.*, 2000). After restoration of isotonicity by the addition of 2ml 1.5M KCl (Merck, Dorset, UK), the homogenate was centrifuged at 250 x g for 10 minutes (to precipitate intact tissue) and the supernatant was decanted into a fresh glass tube. From this supernatant, 100µl volumes were transferred to glass screw-cap culture tubes, to each of which were added 600µl of phosphate-buffered saline (PBS) (Life Technologies, Strathclyde, UK). Triplicate tubes were also prepared as assay blanks containing 100µl of bovine serum albumen (BSA) solution (1mg/ml prepared in PBS) in place of renal homogenate. Each triplicate set of tubes then received 100µl seminal plasma or 100µl PBS (controls and blanks) before being pre-incubated for 30 minutes at 37°C in a gyratory waterbath.

To initiate the 11β-HSD assay, each tube received 100µl $NADP^+$ (4mM in PBS)(Sigma, Dorset, UK) and 100µl PBS containing 0.5µCi [1,2,6,7-^{3}H]-cortisol (Amersham, Aylesbury, Bucks, UK) plus unlabelled cortisol (Sigma, UK) (to a final steroid concentration of 100nM). Tubes were then returned to the waterbath for 60 minutes, after which reactions were terminated by the addition to each tube of 2mls ice-cold chloroform (Merck, UK). To partition the organic and aqueous phases, these tubes were centrifuged at 1000 x g for 30 minutes at 4°C. After aspirating the aqueous supernatant, the organic extracts were evaporated to dryness under nitrogen at 60°C. The steroid residues were resuspended in 20µl ethyl acetate containing 1mM cortisol and 1mM cortisone (Sigma, UK), and were resolved by thin layer chromatography (TLC) using Sigma TLC plates (from UK) in an atmosphere of

homogenate, where protein concentrations were measured using the Biorad protein assay (Bradford *et al.*, 1976; Rosa *et al.*, 1980).

Fractionation of Seminal Plasma by C18 Column Chromatography

Each of the 3 samples of seminal plasma tested in the assays described above were subsequently fractionated using the method previously described by Thurston *et al* (2002a). Aliquots (1ml) of independent seminal plasma samples were applied to separate C18 Sepak cartridges (Amersham, UK) that had previously been conditioned with 20ml methanol and washed with 20ml double-distilled water (DDW). After collecting the loading eluent (i.e. that fraction of the sample not retained by the column), the column was sequentially eluted with 1ml volumes of a stepwise gradient of 0-100% (v/v) methanol (Merck, UK) in DDW. All 1ml fractions were collected into borosilicate tubes and those samples eluted at methanol concentrations greater than 20% (v/v) methanol were evaporated to dryness under nitrogen before being resuspended in 1ml volumes of 20% (v/v) methanol in DDW. Parallel samples of DDW and PBS only were similarly fractionated as negative controls.

Effects of Seminal Plasma Fractions on Renal 11β-HSD Activities

Assays of renal $NADP^+$-dependent 11β-HSD activities were performed as described above with the following modification. Samples were incubated in triplicate in the presence of (a) 100µl of a specific seminal plasma fraction, or (b) 100µl of 20% (v/v) methanol in DDW (i.e. final methanol concentration in 1ml=2%) or (c) 100µl of DDW alone. Enzyme activities in the presence of the 0% and 10% (v/v) methanol fractions were compared to those measured in the controls incubated with DDW alone, whereas enzyme activities in the presence of fractions eluted at ≥20% (v/v) methanol were compared to the 20% (v/v) methanol control.

Spermatozoa Cooling Protocol

As noted above, following collection, semen was diluted into a washing buffer (447mOsm kg-1; Cat: ZS993, IMV, France) to a final concentration of $100x10^6$ spermatozoa/ml, and allowed to cool from 39°C to room temperature at approximately 0.2°C/min. Semen was then further diluted to a concentration of $2x10^6$ spermatozoa/ml with Tyrode's Complete Media (Tyrode's Albumin Lactate Pyruvate; TALP; Parrish *et al.*, 1988) and semen quality was assessed before cooling as detailed below. A total of 1ml of diluted semen (900µl semen plus 100µl seminal plasma or 100µl lipid extract) was cooled at a linear rate from 23°C to 5°C over 100 minutes at a rate of 0.18°C/min using a GP200 waterbath controller (Grant, Cambridge, UK). On reaching 5°C, cooled samples were immediately re-warmed by incubation at 39°C for 10 minutes.

Sperm Cryopreservation Experiments

Following ejaculation, semen was diluted into Beltsville Thawing Solution (BTS; 37g/L glucose monohydrate anhydrous, 6g/L sodium citrate, 1.25g/L sodium hydrogen carbonate, 1.25g/L EDTA-disodium, 0.75g/L KCl, pH 7.2) to a final concentration of $1x10^8$ sperm per ml and allowed to cool from 39°C to room temperature. Fifty millilitres of semen ($1x10^8$/ml) was centrifuged at 15°C for 15 min, at 500*g* and the supernatant containing seminal plasma removed. The sperm pellet was subsequently used for cryopreservation experiments.

For cryopreservation experiments, the sperm pellet was diluted to a concentration of $2x10^6$ spermatozoa/ml in a commercial preservation diluent (Boarciphos: IMV, L'Aigle, France) supplemented with 20% (v/v) seminal plasma fraction [illegible]

nitrogen (Thurston *et al* (2002b). Sperm samples were stored in liquid nitrogen for 24 hours, thawed by plunging into a 40°C water bath for 1min and assessed for sperm survival.

Spermatozoa Viability Assessment

Each semen sample was assessed for sperm quality before and after cooling. Plasma membrane integrity was used as an assessment of sperm viability. Semen was diluted to a concentration of $2x10^6$ spermatozoa/ml with TALP and stained with the fluorescent probes SYBR-14 and propidium iodide according to the manufacturers instructions (Live/Dead Sperm Viability Kit; Molecular Probes Europe, Leiden, The Netherlands)(Garner & Johnson, 1995). Plasma membrane integrity assessments were carried on a Coulter Epics XL instrument flow cytometer (Coulter Corp. Miami, Florida, USA). Flow cytometery set up conditions for SYBR-14 and PI have been validated previously (Medrano, 1998). Three flow cytometry readings were taken from each thawed straw.

Effects of Seminal Plasma on Spermatozoa Viability following Cooling

The effects of the porcine seminal plasma from the 3 independent boars on sperm viability following cooling and re-warming was investigated. Sperm cooling studies and viability assessments were performed as described above. Spermatozoa, isolated from 5 different animals, were cooled and re-warmed in triplicate in the presence of (a) 100µl seminal plasma from the 3 test boars, or (b) 100µl TALP only. Sperm viability assessments for semen cooled in the presence of the test seminal plasma samples were compared to those measured in control aliquots of semen incubated with TALP medium alone.

Extraction of Lipids from Seminal Plasma

The lipid components of each of the 3 samples of seminal plasma tested in the cooling assays described above were concentrated by extraction into

chloroform:methanol as previously described (Eng & Oliphant, 1978). Aliquots (1ml) of the seminal plasma samples were mixed with 5ml of 2:1 (v/v) chloroform:methanol by vortexing for approximately 15 seconds. After centrifugation for 30 minutes at 2000 x g at 4°C, the aqueous layer of each sample was decanted and subjected to two more cycles of chloroform:methanol extraction. Lipids removed from the 3 chloroform phases of the extraction mixture were combined and evaporated to dryness under nitrogen before being resuspended in 1ml volumes of TALP, acetone, DMSO or ethanol (since the solubility of the extracted lipids was unknown). Lipid extracts were stored at -20°C until use.

Effects of Seminal Plasma Lipid Fractions on Spermatozoa Viability following Cooling

Sperm cooling studies and viability assessments were performed as described above with the following modification. Spermatozoa isolated from the ejaculates of 5 independent boars were cooled and re-warmed in triplicate in the presence of (a) 100µl of the test seminal plasma lipid extracts reconstituted in TALP/acetone/DMSO/ethanol, or (b) 100µl TALP/acetone/DMSO/ethanol without seminal lipids. Sperm viability assessments made in semen samples cooled in the presence of the seminal plasma lipid extracts were compared to those measured in the controls cooled in the presence of the relative solvent minus seminal lipids.

RESULTS / CONCLUSIONS

1. 3 samples of boar seminal plasma (prior to C18 fractionation) stimulated $NADP^+$-dependent oxidation of glucocorticoids by 11β-HSD1 within 1 hour in rat kidney homogenates by 68, 76 and 85% (relative to controls).

2. Using C18 column chromatography, it was possible to resolve hydrophobic compounds from seminal plasma that could account for the acute stimulation of $NADP^+$-dependent glucocorticoid oxidation by 11β-HSD1 (Figure 1).

3. The major stimulatory fractions of seminal plasma were resolved at methanol concentrations between 55-75% (v/v) methanol, with a second (minor) stimulatory activity eluted at 100% (v/v) methanol (Figure 1).

4. The degree of stimulation exerted by those hydrophobic fractions of seminal plasma eluted across the range of 55-75% (v/v) methanol varied between seminal plasma samples obtained from different boars (range = 232% to 504% stimulation).

5. The degree of stimulation exerted by those hydrophobic fractions of seminal plasma eluted across the range of 55-75% (v/v) methanol did not correlate with the stimulation of 11β-HSD1 activity by the corresponding seminal plasma samples prior to C18 column chromatography (Table 1).

6. When the samples of seminal plasma were added to spermatozoa (from different boars) to protect against cooling-induced cell death, the ability of different seminal plasma samples to reduce the risk of cell death increased with their content of hydrophobic stimulator(s) of 11β-HSD1 (Table 1).

7. The protective effects of the tested seminal plasma samples were reproduced precisely (in terms of degree of cell protection) by lipid extracts of the seminal plasma extracted into 3 different organic solvents (ethanol, acetone and dimethylsulphoxide) (Figure 2).

8. Taking observations 6 and 7 together implicates the hydrophobic stimulator(s) of 11β-HSD1 as the active hydrophobic components of seminal plasma that protect sperm from cooling-induced death.

9. Addition of the hydrophobic fraction of seminal plasma shown previously to stimulate 11b-HSD1 activity significantly increased the survival of sperm following cooling, cryopreservation and thawing to room temperature (Figure 3).

TABLE 1

Seminal Plasma Sample No.	Stimulation of 11β-HSD1 activity (percentage increase relative to control enzyme activity) by:			Effect on cooling-induced sperm death (percentage of spermatozoa surviving)
	SP prior to fractionation	Hydrophobic SP components eluted at 55-70% methanol	Hydrophobic SP components eluted at 100% methanol	
1	76 ± 6	232 ± 13	85 ± 6	67.5 ± 2.7
2	85 ± 5	341 ± 14	111 ± 5	72.9 ± 0.9
3	68 ± 6	504 ± 13	94 ± 2	79.1 ± 3.1

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CLAIMS

1. A method of determining the suitability of a sample of mammalian semen for cooling and/or cryopreservation, said method comprising:
   (a) providing said sample of semen;
   (b) determining the level of a hydrophobic stimulator of 11β-HSD activity in said sample; and
   (c) assessing, from the level of 11β-HSD stimulator determined, the suitability of the semen sample for cooling and/or cryopreservation.

2. A method according to claim 1 wherein said sample of semen is from a human male.

3. A method according to claim 1 wherein said sample of semen is of rodent, bovine, equine, porcine or ovine origin.

4. A method according to any one of the preceding claims wherein said hydrophobic stimulator of 11β-HSD activity elutes in a fraction from a C18 column at either 50 to 75% or 95 to 100% methanol.

5. A method according to any one of the preceding claims wherein said determination of the level of hydrophobic stimulator of 11β-HSD activity is by contacting said sample of semen with 11β-HSD present in another body fluid or another body derived substance, and determining the effect of the hydrophobic stimulator on the activity of 11β-HSD.

6. A method according to claim 5 wherein said contacting is performed by adding 11β-HSD and a substrate of 11β-HSD to said semen sample.

/

7. A method according to claim 6 wherein said substrate is ^{3}H-cortisol or ^{3}H-corticosterone.
8. A method according to any one of the preceding claims wherein said other body derived substance is a homogenised animal organ.

9. A method according to claim 8 wherein said animal organ is an animal kidney.

10. A method according to claim 9 wherein said animal organ is a rodent organ.

11. A method according to claim 10 wherein said rodent organ is a rat organ.

12. A method according to claim 11 wherein said rat organ is a rat kidney.

13. A method according to any one of the preceding claims wherein a control assay is conducted to allow for any 11β–HSD already present in said sample from said male individual.

14. A method of improving the survival rate of sperm intended for cooling and/or cryopreservation, said method comprising:
    (a) providing a sample of semen; and
    (b) combining said sample of semen with an increased concentration of a hydrophobic stimulator of 11β-HSD activity.

15. A method according to any one of the preceding claims wherein said hydrophobic stimulator of 11β-HSD activity elutes in a fraction from a C18 column at either 50 to 75% or 95 to 100% methanol.

10% by volume, could increase 11β-HSD activity by 100% or more relative to enzyme activity measured in the absence of the stimulator.

17. A method according to any one of claims 14 to 16 further comprising a step of cooling said combination of semen and hydrophobic stimulator of 11β-HSD activity to 5°C or below.

18. A method according to claim any one of claims 14 to 17 further comprising the step of freezing said combination of sperm and hydrophobic stimulator of 11β-HSD activity.

19. A method according to any one of claims 14 to 18 wherein sperm is removed from said sample of semen and said sperm is combined with an increased concentration of a hydrophobic stimulator of 11β-HSD activity.

20. A method according to any one of claims 14 to 19 wherein 85% or more of said human sperm, 40% of said pig sperm, 50% of said horse sperm, 70% of said cow sperm, 50% said sheep sperm or 60% of said rodent sperm survive said cooling and/or cryopreservation.

21. A method of fertilizing an oocyte *in vitro* comprising contacting said oocyte with sperm obtained by a method according to any one of claims 14 to 20.

22. A method of performing an assisted conception/reproductive procedure comprising contacting an oocyte with sperm obtained by a method according to any one of claims 14 to 20 under conditions which allow fertilization of the oocyte.

23. A method according to claim 22 wherein said assisted conception/reproductive procedure is an IVF procedure comprising contacting said oocyte and said sperm *in vitro* and introducing the

fertilized oocyte or zygote or embryo derived therefrom into a female such that it may develop to term.

24. A method according to claim 22 wherein said assisted conception/reproductive procedure is an artificial insemination (AI) procedure.

25. A method according to claim 24 wherein said artificial insemination is an intra-uterine insemination (IUI) procedure.

26. A method of obtaining a hydrophobic product that improves the tolerance of mammalian semen to cooling and/or cryopreservation, comprising the steps of:
    (a) providing a sample of semen;
    (c) removing the seminal plasma from the sperm; and
    (d) fractionating the seminal plasma of (b) to enrich for said product.

27. A method according to claim 26 wherein said seminal plasma is removed from said sperm by centrifugation, Percoll centrifugation or Percoll swim-up.

28. A method according to claim 26 or 27 wherein said fractionating of said seminal plasma is on a C18-methanol affinity chromatography column, TLC, HPLC or FPLC.

29. A product obtainable by fractionation of mammalian seminal plasma and having a stimulatory effect on 11β-HSD activity, which improves the

31. Use of a product as defined in claim 29 or 30 to improve the tolerance of semen to cooling and/or cryopreservation.

32. Use of a product as defined in claim 29 or 30 in the manufacture of a medicament for use in the treatment of inflammatory disease by increasing the survival of topically applied cortisol or cortisol already circulating within the bloodstream.

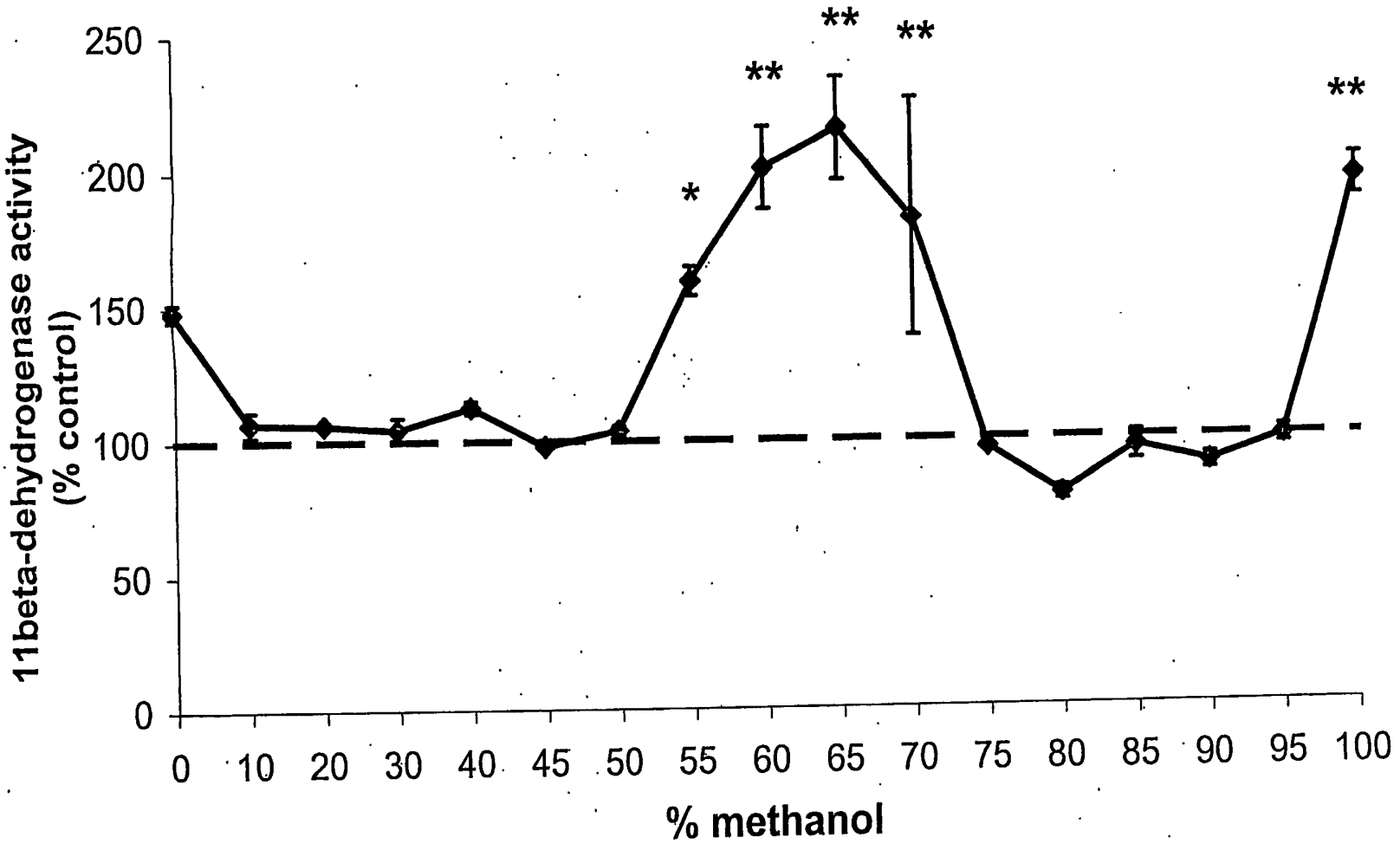
33. Use of a product as defined in claim 29 or 30 in the manufacture of a medicament for use in the treatment of inflammatory disease by stimulating the production of cortisol from circulating cortisone by stimulation of 11β-HSD1.

ABSTRACT

IMPROVEMENTS RELATING TO SEMEN PRESERVATION

The present invention provides a method for determining the suitability of a sample of mammalian semen for cooling and/or cryopreservation, which comprises: (a) providing said sample of semen; (b) determining the level of a hydrophobic stimulator of 11β-HSD activity in said sample; and (c) assessing, from the level of 11β-HSD stimulator determined, the suitability of the semen sample for cooling and/or cryopreservation.

The present invention also provides a method of obtaining a hydrophobic product that improves the tolerance of mammalian semen to cooling and/or cryopreservation, a method of improving the survival rate of sperm intended for cooling and/or cryopreservation and a method of performing an assisted conception/reproductive comprising contacting an oocyte with sperm obtained by a method according to the present invention.


Effect of fractions of seminal plasma eluted from a C18 column with different concentrations of methanol on 11beta HSD activity
11beta-dehydrogenase activity (% control)
250
200
150
100
50
0
0 10 20 30 40 45 50 55 60 65 70 75 80 85 90 95 100
% methanol
*
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**
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FIGURE 1

Variation in the effect of lipids extracted from seminal plasma on sperm viability following cooling to 5 degC and rewarming to 39 degC
(mean+/-SEM; n=5; values with different superscripts differ - $P<0.05$)

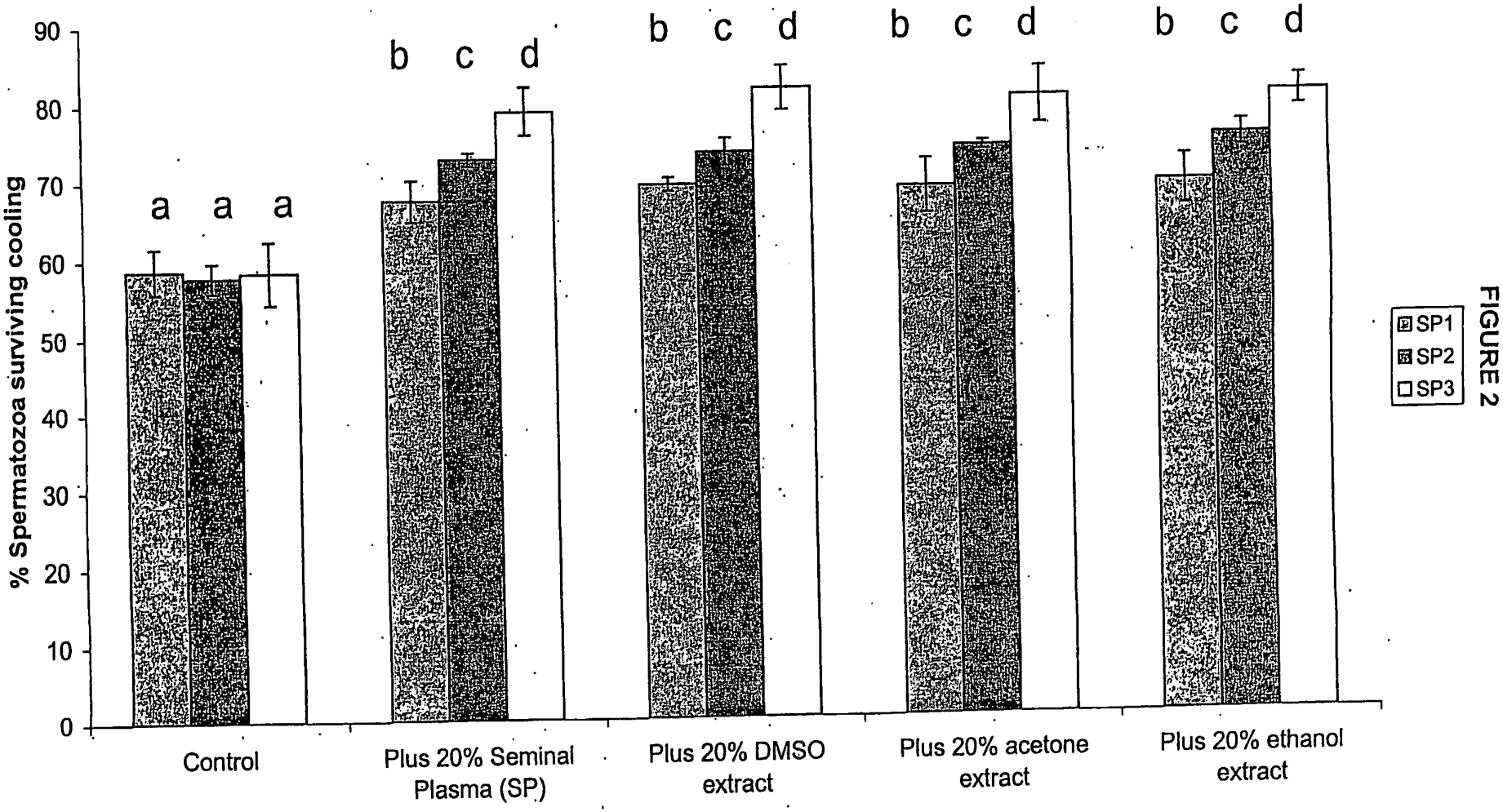
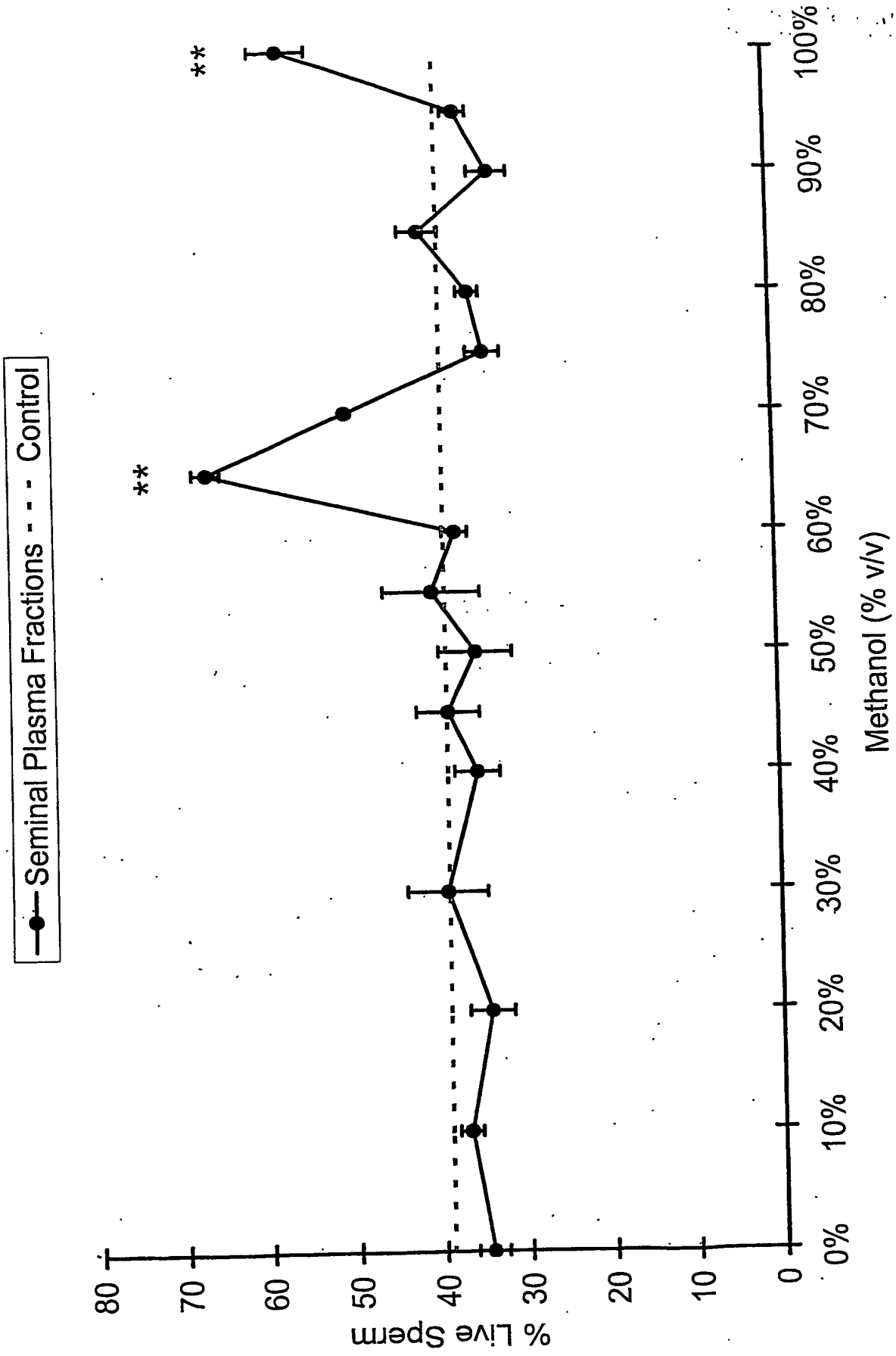


FIGURE 2

FIGURE 3


Seminal Plasma Fractions - - - Control
**
**
% Live Sperm
80
70
60
50
40
30
20
10
0
0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%
Methanol (% v/v)